FEBS Letters 587 (2013) 245–253

FEBS Letters
journal homepage: www.FEBSLetters.org

MeCP2 deficiency is associated with impaired microtubule stability

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ARTICLE INFO

Article history:
Received 10 September 2012
Revised 5 October 2012
Accepted 28 November 2012
Available online 10 December 2012

Edited by Jesus Avila

Keywords:
Rett syndrome
MeCP2
Microtubule
Fibroblast

ABSTRACT

Rett syndrome (RTT) is a neurodevelopmental disorder caused by MECP2 mutations. Previous studies performed on Mecp2-deficient brain showed striking changes in neuronal maturation. We recently showed that MeCP2 deficiency affects microtubule (MT) dynamics in RTT astrocytes. Here, we analyze MT stability in primary fibroblast cultures from patients with RTT syndrome and identify a significant decrease in stability compared to controls. Furthermore, we found that MT stability was reduced both in cells expressing the mutant or the wild-type allele in RTT fibroblasts, suggesting that mutated cells could damage wild-type ones through a non-cell-autonomous pathway. These results suggest that MeCP2 has a stabilizing role on MT dynamics and that its deficiency could lead to impaired MT stability that may explain in part the dendritic abnormalities observed in RTT brains.

1. Introduction

The postnatal neurodevelopmental disorder Rett syndrome (RTT, MIM 312750) is the only one of the five pervasive developmental disorders (including autism) with a known genetic cause (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition). RTT is caused by mutations in the gene encoding methyl-CpG binding protein 2 (MeCP2) [1]. *MECP2* mutations are identified in ~90% and ~50% of cases of typical and atypical RTT, respectively [2]. RTT is characterized by apparently normal development for the first few months followed by loss of neurodevelopmental milestones, similar to what is observed in regressive forms of autism. Clinical features include developmental stagnation and regression, loss of purposeful hand movements and speech, severe autistic features, truncal ataxia, stereotypic hand movements, deceleration of brain growth, autonomic dysfunction and seizures [3]. In addition, whereas *MECP2* mutations seem to be rare in Autism Spectrum Disorders (ASD), *MECP2* mRNA levels were found to be reduced in the absolute majority of ASD cortex [4–7].

Increasing evidence supports the idea that RTT is associated with impairment of dendritic arborization. Several studies described neuronal abnormalities in RTT human brains, and in male Mecp2-mutant mice [8–13]. All these studies suggested that *MECP2* mutation could be involved in cellular mechanisms regulating short term dynamics of dendritic spines early in development within the frame of maximum neural plasticity. However, the underlying cause of the dendritic arborization impairment is largely unknown. Because we and others identified dysregulated expression of proteins involved in regulation of MT dynamics and functions (STMN2 and the ubiquitin-editing enzyme UCHL1, Dynamin I, Dynein I) [14–16], we recently analyzed MT stability and dynamics in mouse Mecp2-deficient cells and showed an alteration of MT dynamics in RTT astrocytes [16]. Recent studies on astrocytes demonstrated that Mecp2-deficient astrocytes detrimentally influence the neuronal dendrite formation in a non-cell-autonomous way [17,18]. MT dynamics alterations may thus contribute to the neuronal dysfunction and to the synaptic plasticity defect, and may explain reduced dendritic branching observed in Mecp2-deficient mice and patients with RTT syndrome [8,9,12,13].

Here we studied microtubule dynamics in human MeCP2 mutant cells, and we report that MeCP2 deficiency affects MT stability and dynamics in fibroblasts from patients with RTT syndrome. Furthermore, we found that MT stability was affected in cells expressing the mutant allele as well as in cells expressing the wild-type allele in human fibroblasts from RTT patient, suggesting that mutated cells could damage wild-type cells through a non-cell-autonomous pathway.

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.11.033

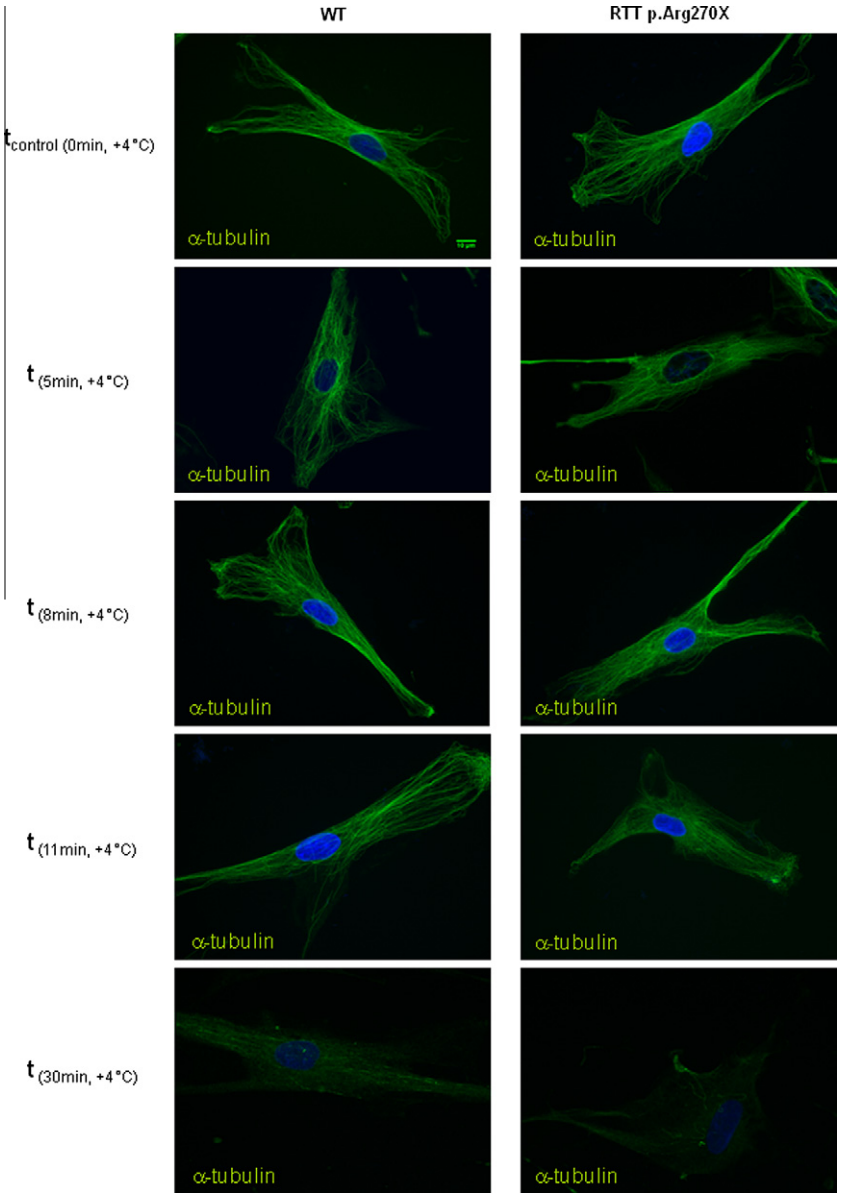

Fig. 1. Effect of cold-induced stress on polymerized MT in fibroblasts from RTT patient (p.Arg270X) and from normal control (WT). Immunofluorescent staining of α-tubulin for WT and RTT fibroblasts after 0, 5, 8, 11, and 30 min at +4 °C. MT from RTT fibroblasts depolymerize faster than MT from WT fibroblasts. After 30 min at +4 °C, MT from both cell cultures appear totally depolymerized.

2. Materials and methods

2.1. Cell culture and single cell cloning for fibroblasts

A skin biopsy was performed on five girls suffering from typical RTT after obtained consent of the parents. Three of them exhibited a non-sense mutation (p.Arg168X; p.Arg255X; p.Arg270X) whereas two harboured a missense mutation (p.Ala2Val; p.Arg306Cys) in *MECP2* gene. For an accurate comparison, our patients had a convergent, classical RTT phenotype and a similar age (8–9 years old). Normal controls were obtained from unaffected females aged 18–20 years. In all studied cases, no X-chromosome inactivation bias was detected in cultured fibroblasts in vitro from 1 to 4 passages. Additional skin biopsies from two patients suffering from atypical RTT and mutated in the *CDKL5* gene (p.Arg79X; and 300 Kb deletion including the *CDKL5* gene) were also studied. Primary fibroblast cultures and clonal cultures were performed as previously described [19,20]. To prevent from epigenetic differences, primary cultures of untransformed fibroblasts with a low number of cell divisions were chosen for our study.

2.2. Microtubule (MT)-depolymerizing and repolymerizing treatments

Human fibroblasts were cultured in Dulbecco's modified Eagle's medium DMEM and supplemented with 10% heat-inactivated fetal bovine serum and 50 units/ml penicillin and streptomycin. All cultures were routinely grown at 37 °C under a 5% CO_2 humidified atmosphere. Concerning MT-depolymerizing treatment, cells on sterile glass coverslips were incubated in the refrigerator (4 °C) for various time intervals (0, 5, 8, 11, and 30 min) before washing them twice in cold PBS and fixing them in ice-cold methanol for 7 min at −20 °C. For reversibility study, in order to induce MT-repolymerization, after a 30-minutes cold treatment, cells were incubated at 37 °C for various time intervals (0, 4, and 8 min) before washing them twice in PBS and fixing them in ice-cold methanol for 7 min at −20 °C.

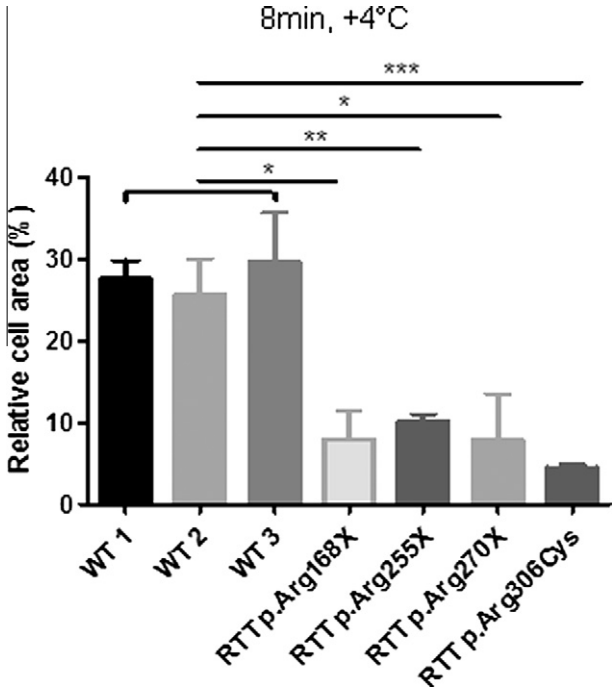

Fig. 2. Effect of cold-induced stress on polymerized MT (PMT) in fibroblasts from patients with RTT syndrome and from normal controls after 8 min at +4 °C. This graph represents the relative cell area occupied by the microtubule network (ratio between the area occupied by PMT to the whole cell area) for WT (WT1, WT2 and WT3) and RTT fibroblast cultures bearing different types of *MECP2* mutations such as non-sense mutations (p.Arg168X; p.Arg255X; p.Arg270X) and missense mutations (p.Arg306Cys). After 8 min at +4 °C, MeCP2-deficient fibroblasts MT are almost totally depolymerized whereas normal controls still show several PMT.

2.3. Sedimentation assay

Quantification of cellular tubulin in microtubules and tubulin pools was performed using the microtubules/tubulin in vivo assay kit (Cytoskeleton, Denver, Colorado, USA) according to the manufacturer instructions. In brief, cells were incubated in lysis buffer in the presence of 100 μM GTP, and centrifugated at 100 000×*g* for 30 min in an TLA45 rotor in a Beckman ultracentrifuge (Beckman instruments, Palo Alto, CA). Amount of free tubulin and microtubules in supernatant and pellets was determined by Western blot using the tubulin antibody provided at 1:1000 dilution.

2.4. Antibodies

The primary antibodies used for semi-quantitative or qualitative studies were: antibody against the C-terminal domain of MeCP2 (amino acids 465–478) (M9317, Sigma, Lyon, France, 1/1000), anti-α tubulin (Sigma T9026, 1/1000), anti-acetylated tubulin (Sigma M6793, 1/2000), anti-tyrosinated tubulin (Sigma T9028, 1/1000). For normalization, anti-GAPDH (Santa Cruz Biotechnology, Heidelberg, Germany, 1/2000) have been used.

2.5. Western blotting

Briefly, proteins were extracted from the fibroblasts cultures, by Laemmli sample buffer two times (BioRad, Richmond, CA, USA). The protein samples were separated by a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to Hybond-C nitrocellulose membrane (GE Healthcare). After blocking non-specific sites by a TBS–Tween 20 0.05%/fat milk 5% solution, 1 h at room temperature, the membrane was incubated overnight at 4 °C with primary antibody, washed and incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-goat IgG (dilution 1:5000) and developed with the enhanced chemiluminescence method (ECL; GE Healthcare).

2.6. Immunostaining

Cells were washed twice with PBS and fixed with methanol at −20 °C during 7 min, incubated with appropriated antibodies and then mounted with Vectashield mounting medium with DAPI (Vector Laboratories) and analyzed with a Leica DMRA2 fluorescence microscope. Quantitative analysis of microtubules was carried out with a Leica DMRA2 fluorescence microscope (Leica, Rueil-Malmaison, France) and included measurement of their fluorescence using the MetaMorph soltware and analyzis of digital images collected with a digital CCD camera. For the depolymerization quantitative analyzis, extended focus images of a single well-spread cell per view with minimal thickness were used. Microtubule subpopulations in the area of interest were computed by the original image segmentation with threshold set of 200% of background level and by calculating the percentage of above-the-threshold pixels. The relative area occupied by the microtubule network in cell compartment was calculated. The ratio between the area occupied by MT to the whole cell area was determined. In each experiment and for each condition, at least 30 cells were quantitatively analyzed. Repolymerization was quantified as the percentage of cells with centrosomal MT asters after 4 min incubation at 37 °C. Normal fibroblasts from control individuals were included in each experiment. All individual experiments were performed in triplicate.

2.7. Drug

For microtubule stabilization, fibroblasts were incubated for 30 min either in the absence of drug or with 250 nM paclitaxel (Taxol, Sigma T1912) and then submitted to cold-induced depolymerization during 11 min at 4 °C.

3. Results

3.1. MeCP2 deficiency affects the rate of MT depolymerization during cold-induced catastrophe

We first investigated whether MT stability is affected by MeCP2 deficiency. To this end, we performed a cold-induced MT depolymerization in primary cultures of human fibroblasts obtained from patients with RTT syndrome and analyzed by immunofluorescent staining of α-tubulin the cytoskeleton morphology at various times of cold treatment. We clearly observed that MeCP2 deficiency affects the rate of MT depolymerization and that MeCP2-deficient fibroblasts displayed a more pronounced response to cold-induced stress (Fig. 1). After 8 min at +4 °C, MT from RTT fibroblasts are almost totally depolymerized whereas normal controls still show several polymerized MT (Fig. 1). The same results were obtained with human RTT fibroblasts bearing different types of *MECP2* mutations such as non-sense mutations (p.Arg168X; p.Arg255X; p.Arg270X) and missense mutations (p.Arg306Cys) (Fig. 2).

Because *MECP2* is an X-linked gene that undergoes X-chromosome inactivation in females, each cell expresses *MECP2* exclusively from one of the two X chromosomes (the active X), while the copy on the inactive X chromosome is silenced. In order to separate cells that express the wild type from those that express the mutant *MECP2* allele, we performed single-cell cloning of fibroblasts isolated from skin biopsy from a RTT patient expressing exclusively the p.Arg270X mutant allele. We again observed that MT from MeCP2-deficient fibroblasts bearing the p.Arg270X muta-

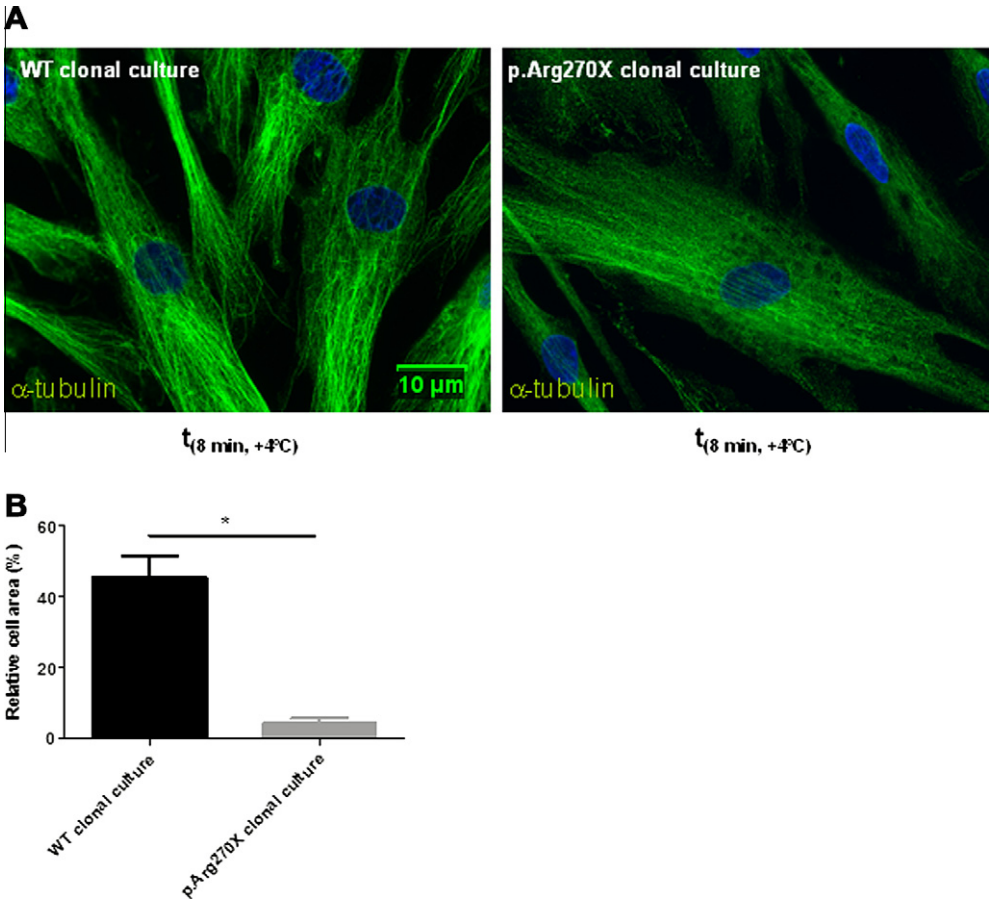

Fig. 3. Effect of cold-induced stress on polymerized MT (PMT) in fibroblasts clonal primary cultures from p.Arg270X mutation patient. (A) Immunofluorescent staining of α-tubulin for WT and mutant clonal culture (p.Arg270X clonal culture) after 8 min at +4 °C. (B) Graph representing the relative cell area occupied by the microtubule network for WT clonal culture and p.Arg270X clonal culture. After 8 min at +4 °C, the clonal culture expressing exclusively the WT allele (WT clonal culture) still shows several PMT whereas the mutant clonal culture (p.Arg270X clonal culture) already shows totally depolymerized MT.

tion displayed a more pronounced response to cold-induced stress as compared to wild-type fibroblasts from the same individual (Fig. 3).

To confirm our data by an other approach, we quantified microtubules and tubulins pools by Western blot after ultracentrifugation. After 15 min at +4 °C, polymerized tubulins tend to be reduced in RTT fibroblasts compared to normal controls (Fig. 4).

All these data suggest that MT from RTT cells are more sensitive to the depolymerizing effect of cold temperature than those of WT cells.

3.2. MeCP2 deficiency affects MT repolymerization after cold-induced catastrophe

Next we investigated a temperature-induced MT repolymerization. After complete depolymerization at 4 °C for 30 min, cells were shifted back to 37 °C for various times (0, 4, and 8 min). The rate of MT regrowth from the centrosome was noticeably lower in MeCP2-deficient fibroblasts. After 4 min at 37 °C, centrosomal MT asters appeared in nearly 100% of wild-type cells, but only in approximately 20% of MeCP2-deficient cells (Figs. 5 and 6). Taken together, all these results are consistent with reduced MT stability in RTT cells harboring a *MECP2* mutation.

3.3. MeCP2 has a cell- and a non-cell autonomous effect on MT stability

Using a specific MeCP2-antibody directed against the C-terminal domain of the MeCP2 protein, we distinguished RTT fibroblasts

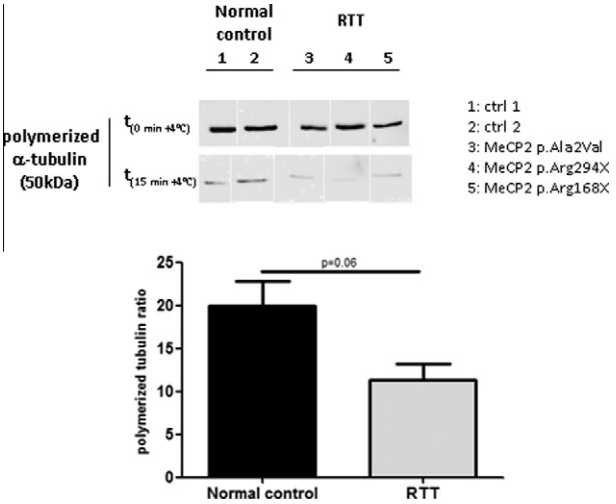

Fig. 4. Effect of cold-induced stress on polymerized MT (PMT) in fibroblasts from patients with RTT syndrome (lanes 3–5, 3: p.Ala2Val; 4: p.Arg294X; 5: p.Arg168X) and from normal controls (lanes 1 and 2) after 15 min at +4 °C. Quantification of polymerized tubulin was performed by Western blot after centrifugation at 100000×*g* for 30 min. The graph represents the polymerized tubulin ratio before and after cold treatment.

expressing the wild-type allele from RTT fibroblasts expressing the mutated allele (non-sense mutation). Although it appeared to be more pronounced in MeCP2-deficient cells, MT stability was also

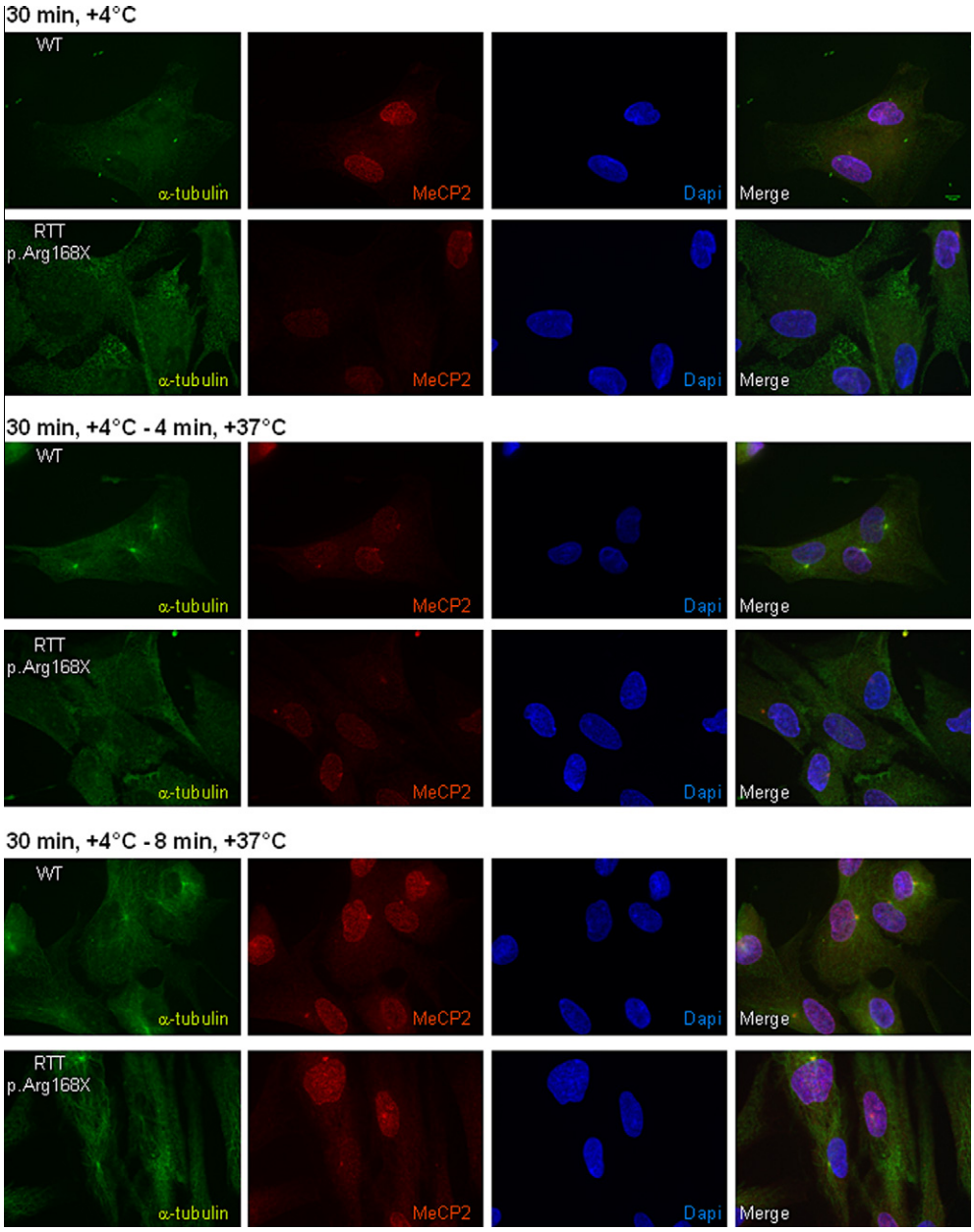

Fig. 5. Effect of MeCP2 deficiency on tubulin repolymerization after cold-induced catastrophe. Immunofluorescent staining of α-tubulin for WT and RTT fibroblasts (p.Arg168X): cells are submitted to a MT repolymerization step following total depolymerization (0, 4, and 8 min at +37 °C after 30 min at +4 °C).

reduced in cells expressing the wild-type allele in human fibroblasts from RTT patient, suggesting that mutated cells could damage wild-type cells through a non-cell-autonomous pathway (Fig. 6). The same observations were obtained in MT depolymerization studies in human RTT fibroblasts.

3.4. CDKL5 deficiency does not affect MT stability

Considering the similar phenotypes caused by mutations in *MECP2* and *CDKL5*, an X-linked gene involved in atypical form of RTT, it has been suggested that the two genes could play a role in a common pathogenic process [21–24]. It has been shown that CDKL5 is a protein whose expression in the nervous system overlaps with that of MeCP2 during neural maturation and synaptogenesis. Down-regulating CDKL5 by RNA interference (RNAi) in cultured cortical neurons inhibited neurite outgrowth and dendritic arborization development through a cytoplasmic mechanism, whereas over-expressing CDKL5 had opposite effects [23]. To find if the cellular consequences of the deficiency of these two proteins are similar, we performed the same cellular approaches in fibroblasts from two atypical patients with RTT syndrome bearing *CDKL5* mutations (p.Arg59X mutation; deletion of exon 16 of the *CDKL5* gene). We did not observe any significant difference either in terms of MT depolymerization when cells were incubated at 4 °C, nor in terms of repolymerization after cells were shifted back to 37 °C (Fig. 7). These results suggest that MeCP2 and CDKL5 may probably not share the same molecular and cellular pathway and that MT stability impairment described above is likely to be specific to a MeCP2 deficiency.

3.5. MeCP2 deficiency affects acetylated but not tyrosinated MT dynamics

In 2004, Matarazzo and Ronnett found evidence of temporal and regional proteomic pattern differences between wild-type and Mecp2-deficient mice, and conclude that not only transcrip-

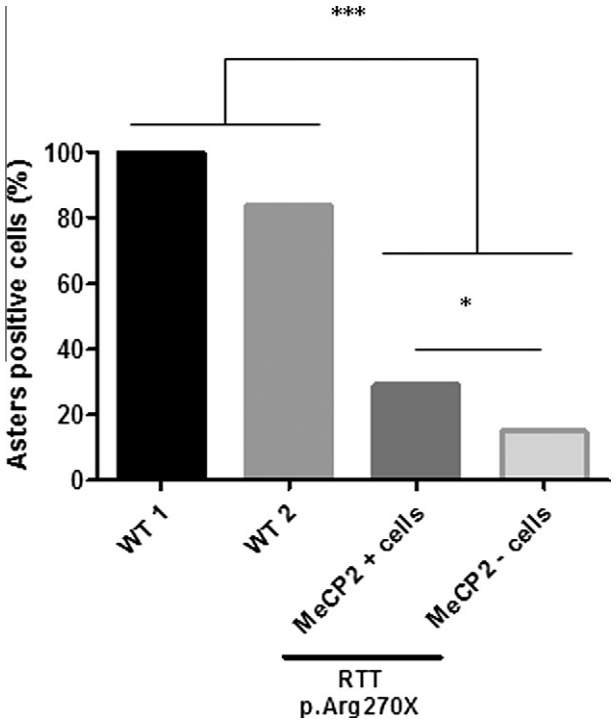

Fig. 6. Quantification of MT repolymerization as the percentage of cells with centrosomal MT asters after 4 min of incubation at 37 °C in WT and RTT (p.Arg270X) fibroblasts. Cells from RTT culture display significantly less repolymerized MT as compared to control (WT1 and WT2). Within the RTT fibroblast culture, MeCP2 deficient cells (MeCP2 − cells) show two times less repolymerized MT than those expressing the WT allele (MeCP2 + cells), suggesting that MeCP2 has a cell- and a non-cell-autonomous effect on MT dynamics.

tion should be taken into account as a source for the changes in protein expression, but posttranslational protein modifications as well [25]. We speculated that MeCP2 may be involved in posttranslational modification of tubulin, and in MT stability modification, although the precise relationship between these tubulin modifications and MT stability has not been elucidated. Usually, acetylated tubulins are known to exist in stable, long-lived MT whereas tyrosinated tubulins are present in labile and neoformed MT [26]. To investigate whether the faster MT depolymerization in MeCP2-deficient cells is related to a change of acetylated and/or tyrosinated tubulin level, we carried out Western blot analysis, which allow estimating the total pool of tubulins (i.e. soluble + polymerized) at two points of our depolymerisation kinetics: 0 and 8 min at +4 °C. During depolymerization, a marked decrease of acetylated tubulin without any changes in total α-tubulin and tyrosinated tubulin was observed (Fig. 8A). Interestingly, although we observed a heterogeneity likely due to X chromosome inactivation status, the quantification of this deacetylation in WT and RTT cultures during depolymerization show that tubulins from RTT fibroblasts are faster deacetylated than tubulins from the WT culture (Fig. 8B). These data are consistent with the reduced MT stability observed above.

3.6. Taxol corrects the defect of MT stability in MeCP2-deficient cells

To investigate the ability of Paclitaxel (taxol), a common MT stabilizer, to correct the impaired cellular phenotype observed during cold-induced stress on RTT fibroblasts, cells were incubated in the absence of drug or with 250 nM taxol for 30 min, and then subjected to depolymerization 11 min at 4 °C. As expected, after 11 min at 4 °C in the absence of taxol, MT from RTT fibroblasts are significantly more depolymerized than the WT control, thus confirming again the MT stability impairment in the MeCP2-deficient cells. After a 30 min treatment with 250 nM taxol, RTT fibroblasts no longer show difference in terms of rate of depolymerization, i.e. in terms of cold-stress sensitivity. Thus, it can be postulated that taxol is able to correct the faster MT depolymerization observed on RTT fibroblasts, at least in our experimental conditions (Fig. 9).

4. Discussion

Increasing evidence supports the idea that Rett syndrome is associated with impairment of dendritic arborization. Several studies described neuronal abnormalities in RTT human brains and in male Mecp2-mutant mice [8–13]. All these studies suggested that *MECP2* mutation could be involved in cellular mechanisms regulating short term dynamics of dendritic arborization early in development within the frame of maximum neural plasticity. However, the underlying cause of the dendritic arborization impairment is largely unknown. Although actin and its regulatory proteins play a central role in morphogenesis of dendritic spines, recent studies

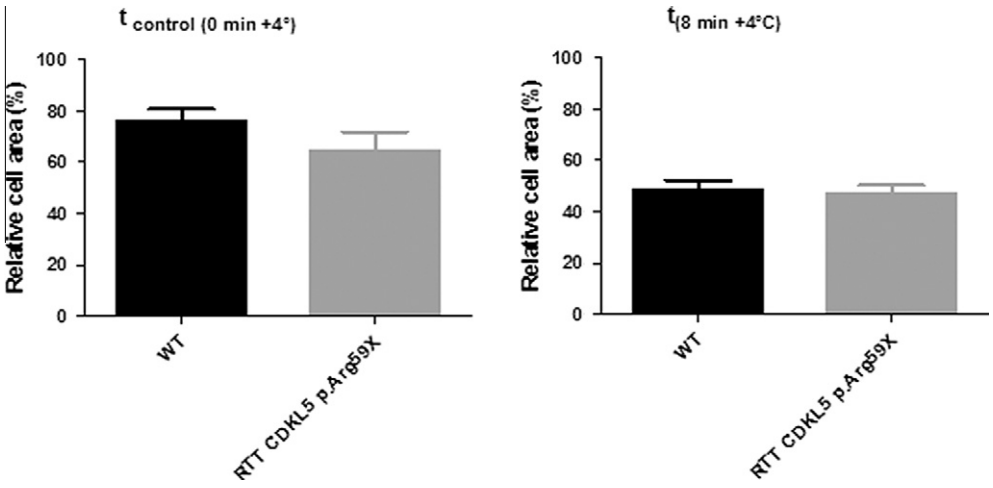

Fig. 7. Effect of cold-induced stress on polymerized MT (PMT) in RTT fibroblasts primary culture from a *CDKL5* mutation patient (p.Arg59X mutation). After 8 min depolymerization at +4 °C, the culture from the *CDKL5* mutation patient shows no difference in the rate of PMT as compared to the wild-type (WT) culture, suggesting that *CDKL5*-mutated fibroblasts do not exhibit increased sensitivity to cold-induced stress.

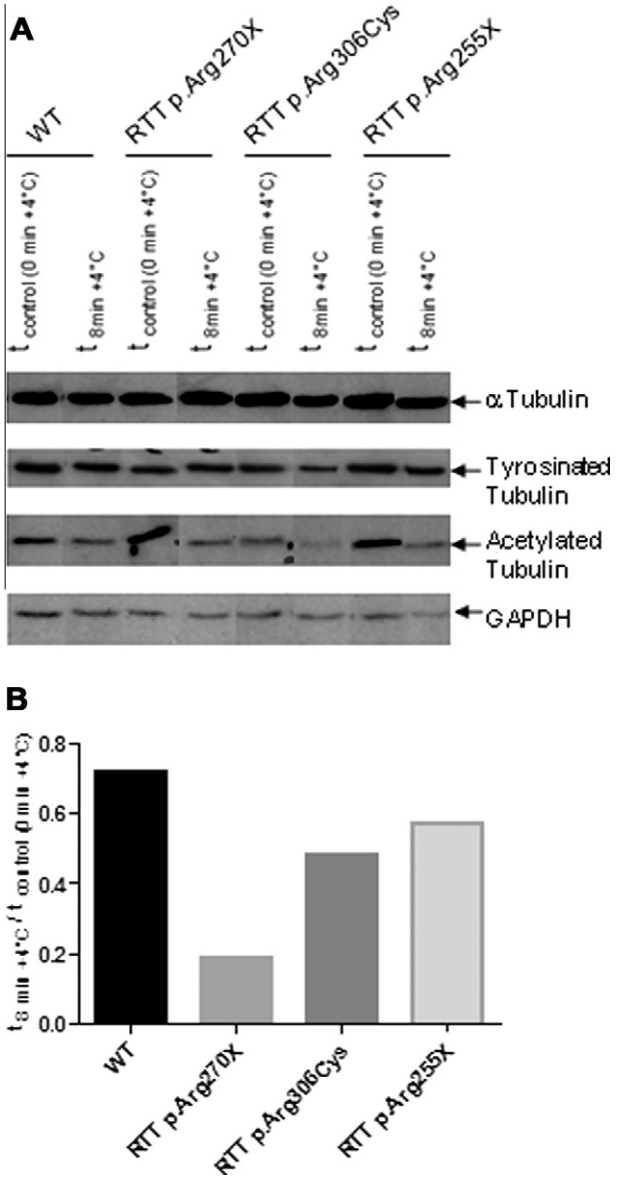

Fig. 8. (A) Western blot analysis of total α-tubulin, tyrosinated tubulin and acetylated tubulin in wild-type (WT) and fibroblasts from patients with RTT syndrome (p.Arg270X; p.Arg306Cys; p.Arg255X) before and after 8 min depolymerization at +4 °C. A marked decrease of acetylated tubulin is observed during the depolymerization kinetics without any changes in total α-tubulin or tyrosinated tubulin. (B) Quantification of the decrease of acetylated tubulin during depolymerization. During depolymerization, tubulins from MeCP2 mutated fibroblasts are markedly deacetylated as compared to the WT culture, which is deacetylated to a lesser extent.

have revealed that dynamic microtubules are indispensable for the maintenance of mature dendritic spine morphology [27]. Because we and others identified dysregulated expression of proteins involved in regulation of MT dynamics and functions (STMN2 and the ubiquitin-editing enzyme UCHL1, Dynamin I, Dynein I) [14,15,19], we previously analysed MT dynamics in brain using two different RTT mouse models and showed an alteration of MT dynamics in RTT astrocytes [16].

In this report, we showed that human fibroblasts from patients with RTT syndrome exhibit increased sensitivity to cold-induced stress as compared to WT controls, suggesting that *MECP2* mutation is associated with reduced MT stability. The same results are obtained with fibroblasts carrying different types of mutations, suggesting that MT dynamics impairment is not dependent on the mutation type. Because *MECP2* is located on the X chromosome, primary fibroblast cultures from female patients with RTT syndrome result in a mosaic for loss of MeCP2 function consisting of cells that express the wild type *MECP2* from the active X and cells that express the mutant *MECP2* from the active X. To eliminate possible changes resulting from inter-individual genetic differences, we compared MT stability in clonal cells originating from the same RTT patient, and we also observed that clonal culture expressing exclusively the *MECP2* mutant allele displayed a more pronounced response to cold-induced stress than clonal culture expressing exclusively the WT allele, thus confirming that MeCP2 deficiency may lead to reduced MT stability.

Using a specific antibody directed against the C-terminal part of the protein, we compared MT stability in RTT cells expressing the wild-type allele (MeCP2+) and RTT cells expressing the mutant allele (non-sense mutation) (MeCP2−), and interestingly, although MeCP2 − cells are more affected than MeCP2 + cells, all cells from a RTT patient presented MT stability abnormalities, suggesting that wild-type cells are likely to be affected in a non-cell-autonomous fashion by the mutant cells. This notion is in accordance with the fact that, in heterozygous human patients, the majority of pyramidal cortical neurons show aberrant dendritic morphology [8]. Considering the similar phenotypes caused by mutations in *MECP2* and *CDKL5*, an X-linked gene involved in atypical form of RTT, it has been suggested that the two genes could play a role in a common pathogenic process [23,24]. Interestingly, it has been shown that the two proteins are widely co-expressed in the brain and are similarly activated during neuronal maturation and synaptogenesis [28,29]. At the molecular level, the two proteins interact together and CDKL5 mediates MeCP2 phosphorylation at least in vitro [23,24]. There are also reasons to believe that MeCP2 also performs CDKL5-independent functions. When we performed the cold-induced repolymerization experiments on human fibroblasts carrying mutations in the *CDKL5* gene, we did not observe any changes in MT stability. We conclude that MeCP2 status, but not CDKL5, is associated with microtubule stability.

One explanation of the MT dynamics impairment in MeCP2-deficient cells may be linked to abnormal post-transcriptional modifications of tubulins. We investigated this using specific antibodies directed against acetylated and tyrosinated tubulins, and observed a faster deacetylation in the MT from patients with RTT syndrome as compared to WT. Supporting this notion is the finding that, inhibition of the N-acetyltransferase complex (consisting of N-acetytransferase 1 (NAT1) and arrest defective 1 (ARD1)), which mediates tubulin acetylation results in less dendritic branches in cultured Purkinje neurons [30]. However, our results contrast with a previous study which reported reduced levels of Tyr-α-tubulin and Glu-α-tubulin proteins in cerebral cortex extracts from patients with RTT syndrome with almost no decrease in Δ2 α-tubulin and total α-tubulin associated with reduced expression of TUBA1B, TUBA3 (renamed TUBA1A) and TUBA4A. The molecular mechanism that brings about reduced expression of the *TUBA3* gene remains still obscure [31]. This apparent discrepancy may be explained by the fact that we and other did not observe dysregulated expression of tubulin genes either in fibroblasts from human RTT patient nor in human brain [32,33].

Taken together, our results using biochemical and cellular approaches indicate that MeCP2 deficiency is associated with an alteration of MT dynamics in a cell-dependent way. RTT cells exhibit increased sensitivity to cold-induced stress as compared to wild-type controls in a non-cell-autonomous fashion. It is likely that the increased sensitivity to cold-induced stress observed in our experiments is the consequence of the global chromatin regulator function of MeCP2 suggested by many authors [33]. These data are consistent with recent findings suggesting that the selec-

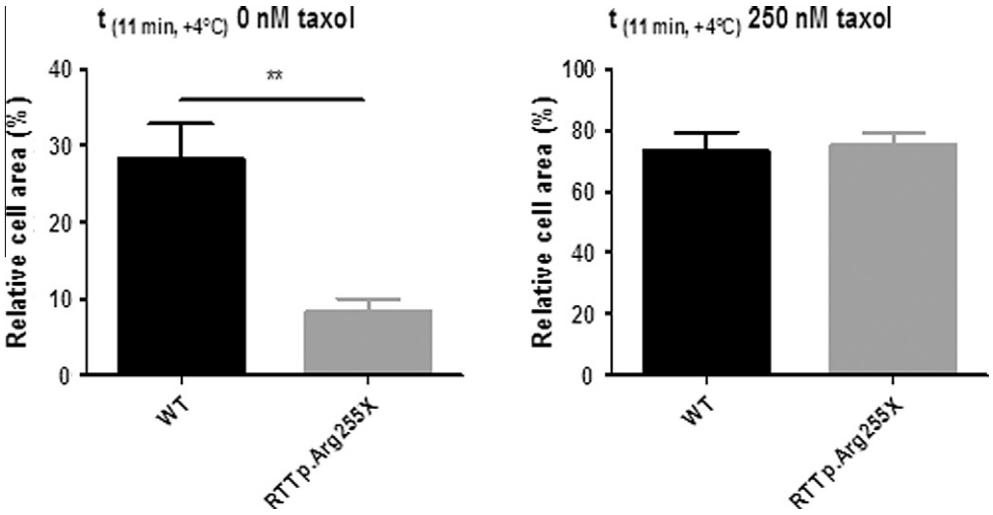

Fig. 9. Effect of Taxol on MT dynamics of MeCP2-deficient cells. Cells were incubated in the absence of drug or with 250 nM taxol for 30 min, and then subjected to depolymerization 11 min at 4 °C. After a 30 min treatment with 250 nM taxol, RTT fibroblasts no longer show difference in terms of rate of depolymerization, i.e. in terms of cold-stress sensitivity. Taxol corrected the MT depolymerization defect observed on RTT fibroblasts. WT (wild-type).

tive loss of Mecp2 in non-neuronal cells such as astrocytes elicits, at least in part, an RTT-like phenotype [34]. Identifying MT dynamics abnormalities in human neurons and astrocytes generated from RTT induced pluripotent cells is an important area of future investigation. The involvement of microtubule dysfunction in RTT suggests that it may be possible to amend RTT using microtubule targeting drugs. In this study, we found that taxol, a drug acting against microtubule depolymerisation through its binding to β-tubulin, is able to correct microtubule dynamics alterations in MeCP2-deficient cells. Our data provide the first evidence that a microtubule stabilizer might have a beneficial effect, opening new possibilities for the development of novel pharmacological approaches for the treatment of Rett syndrome.

Funding

This work was supported in part by Institut National de la Santé et de Recherche Médicale (ANR e-Rare Euro-RETT) and Fondation Jerome Lejeune. The authors greatly acknowledge the Cochin Imaging Facility.

Acknowledgments

We thank the families for participating, especially the members of the Association Française du Syndrome de Rett (AFSR). The authors thank Karine Poirier, Yoann Saillour for valuable discussions about the results and for technical assistance.

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